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SPECIES- AND SITE-SPECIFIC EFFICACY OF COMMERCIAL BIOCIDES AND APPLICATION SOLVENTS AGAINST LICHENS

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36 **Abstract**

37 Control of lichens on stone cultural heritage is mostly achieved by a combination of mechanical
38 removal with biocide applications. However, there is a lack of scientific evidence on the efficacy of
39 different biocides on different species, and on the consistency of biocide effects on heritage sites in
40 different environmental conditions. This results in some uncertainty when conservation
41 interventions to control lichens are routinely defined on the basis of restoration tradition or
42 empirical evaluation, without experimental measures of how lichens respond. In this work, we
43 quantitatively evaluated (a) the efficacy of five commercially-available biocides, applied using a
44 brush or with a cellulose poultice, against two species (*Protoparmeliopsis muralis*, *Verrucaria*
45 *nigrescens*), and (b) whether the effects on the two species were consistent, per treatment, across
46 three Italian heritage sites. Lichen vitality was quantified through analyses of chlorophyll *a*
47 fluorescence (Chl_aF) and ergosterol content. The results indicated that all the tested biocides, and
48 their organic solvents, affected the vitality of both the species. However, most of treatments
49 displayed different efficacy on each species, across the different sites and between brush and
50 poultice applications. Accordingly, when a conservation intervention to control lichen growth is
51 planned, biocide treatments need both species- and site-specific calibrations and lichen vitality
52 should be properly ascertained *in situ* by monitoring Chl_aF parameters (F_V/F_M and F_0) twenty days
53 after trial biocide applications.

54

55 **Keywords**

56 biocide; chlorophyll *a* fluorescence; ergosterol; lichen; organic solvents

57

58

1. Introduction

The effects of lichens on stone monuments are nowadays considered a matter of debate, as researchers are increasingly contributing, and counterposing, evidence for lichen-related biodeterioration and bioprotection processes (Salvadori and Casanova-Municchia 2016). The need to remove lichens in all cases may be reasonably questioned, as for example in cases where lichen colonization accounts for a negligible deterioration effect, shows some bioprotective attributes, contributes to the aesthetic of the monument and/or represents biodiversity value (Pinna 2014). Nevertheless, in cultural heritage management a direct relationship between lichens and weathering is still usually envisaged, and lichen removal is generally planned as component of restoration interventions (Caneva et al. 2008).

In any cleaning interventions, devitalization of lichens is necessary to avoid them being undesirably scattered, rather than controlled, by the cleaning actions (Caneva et al. 2008). So far, the application of biocides has been the most followed approach to kill lichens, although chemical treatments give rise to concerns about their impact on the environment (e.g. Gromaire et al. 2015) and have already showed technical limitations (Speranza et al. 2013 with refs therein). Biocide application has indeed yielded mixed results, including poor treatment response, changes in community dynamics, persistence of dead thalli, and damage to substrate surfaces (Seaward 2015). Accordingly, several innovative and promising approaches have been proposed in the last years to substitute for, or reduce, biocide application, including heat shock treatments (Tretiach et al. 2012), infrared and ultraviolet laser irradiation (Speranza et al. 2013; Sanz et al. 2015; Pozo et al. 2016), and others, which still need to be better calibrated on lichens, such as anatase photocatalysis (Fonseca et al. 2010) or enzymatic treatments (Scarpa et al. 2016). Nevertheless, the adoption of these new techniques is generally limited by experimental time, extent of surfaces to be treated, and, in some cases, economic constraints, while the use of biocides persists as a routinely adopted approach, with protocols often based on traditions and empirical evaluations more than on experimental analyses of their efficacy in each case-study (Caneva et al. 2008).

Research on biocidal effects on lichens has been conducted since the 1970s and 1980s, with treatment success being mostly empirically defined *in situ* (Caneva et al. 1996, and references therein), while standardization of experimental techniques to assess lichen devitalization after biocide application (i.e. fluorescence microscopy) was established at the beginning of 1990s (Normal 1994). Conservators have claimed some difficulties in directly testing a range of biocide and cleaning agents (Schnabel 1991), and have noted the need for comprehensive reviews on commonly used biocidal materials (e.g. Caneva et al. 1996). However, as a response, lists of products rather than investigations into their efficacy have been produced, and some products have become outdated over the years, following the recognition of their toxicity-related environmental and health hazards (Nugari and Salvadori 2003; European-Commission-Regulation 2007; SCENIHR 2009). More recent research has considered the biocidal effect(s) of restricted sets of products (e.g. Tretiach et al. 2007; de los Ríos et al. 2012), in comparison with physical treatments (e.g. Fonseca et al. 2010; Tretiach et al. 2012) or in combination with other restoration products (e.g. Pinna et al. 2012). Different approaches to assess the effects of the treatments have been considered, including microscopical observation of chlorophyll epifluorescence in photobionts (Nugari et al. 1993), SEM evaluation of the integrity of anatomical structures of both lichen

101 partners (Speranza et al. 2012), fluorimetric analyses of biophotonic activity (Bajpai et al. 1992)
102 and chlorophyll *a* fluorescence of photobionts (Chl_aF) (Tretiach et al. 2008, 2010), electrical
103 conductivity of thalli (Cuzman et al. 2013) and molecular assessments (e.g. DGGE; Cámara et al.
104 2011). The diversity of methods used to assess lichen devitalization in these studies makes it hard to
105 compare results. Moreover, although a species-specific lichen sensitivity to biocides has been
106 suggested (Alstrup 1992; Nimis and Salvadori 1997), only few researchers have included a focus on
107 this feature (Tretiach et al. 2007, 2010, 2012). More remarkably, researchers have neglected to
108 evaluate the *in situ* reproducibility of devitalization results across different heritage sites, nor have
109 they clarified if different biocidal approaches, in terms of active principle, preparation solvent
110 and/or application method, may be more or less suitable against certain species, on certain stone
111 substrates or under certain macro- and micro-climatic conditions. However, similar information, in
112 parallel with research on alternative approaches for lichen control, would be of value to optimize
113 routinely-adopted biocidal application, and, consequently, reduce related environmental
114 contamination (Scheerer et al. 2009).

115 In this research, we compared the effects of five commercial biocides, nowadays widely used in
116 Europe (BiotinR, BiotinT, DesNovo, Lichenicida 464, Preventol RI80), and their application
117 solvents (water, acetone, White Spirit) on the vitality of two epilithic lichens [*Protoparmeliopsis*
118 *muralis* (Schreb.) M. Choisy and *Verrucaria nigrescens* Pers.] commonly found on stone cultural
119 heritage in Europe and beyond (Nimis et al. 1992). The effects of the herbicide glyphosate (Glifene
120 SL) and of the lichen secondary metabolite usnic acid, having biocidal potential against other
121 deteriorogenic lithobionts (Gazzano et al. 2013), were also assayed. All the products were applied *in*
122 *situ*, with single brush and poultice applications at concentrations following the producers'
123 recommended ranges, on lichen thalli growing on sedimentary rocks in three Italian heritage sites
124 located in different (phyto-)climatic areas (as defined in Nimis and Martellos 2008). The research
125 did not aim to rank the performance of the different products, as each product was not tested in all
126 possible concentrations, application methods and treatment cycles. The aims of the study were to
127 quantify, for a series of biocide treatments, (a) if each approach (i.e. biocide × application method)
128 showed a similar efficacy against different lichen species, and (b) if efficacy results were consistent,
129 per species per treatment, between different sites. To accomplish these aims, we examined in each
130 study site the vitality of lichen thalli before and after the treatments in terms of chlorophyll *a*
131 fluorescence (Chl_aF) of the photobiont, recognized as an ideal tool for checking the vitality of
132 photosynthetic organisms, including lichens (Tretiach et al. 2012; Malaspina et al. 2014).
133 Additional analyses were also, in turn, performed to clarify the lichen response to biocide
134 treatments, including microscopic assessment of chlorophyll epifluorescence in photobionts and the
135 assessment of mycobiont vitality in terms of ergosterol content.

136

137 **2. Materials and methods**

138 **2.1 Sites and lichen species**

139 Biocide applications on lichens were performed, *in situ*, at three heritage sites distributed in
140 different (phyto-)climatic areas of Italy: (A) the Roman Archaeological site of Industria [Monteu da
141 Po, Torino; UTM ED50, N 5001078, E 422890; 170 m], in the dry sub-Mediterranean area; (B) the
142 Roman Archaeological site of Luni [Ortonovo, La Spezia; UTM ED50, N 4879338, E 581882; 3

m], in the humid Mediterranean area; (C) the Boboli Gardens [Firenze; UTM ED50, N 4847851, E 680788; 49 m], in the humid sub-Mediterranean area (Fig. S1). Treatments were performed on mature thalli of the epilithic crustose placodiomorph *Protoparmeliopsis muralis* (Schreb.) M. Choisy and the epilithic crustose areolate *Verrucaria nigrescens* Pers. (Fig. S1), which were identified following Smith (2009). These two subcosmopolitan species are extremely common both in urban and natural habitats (Nimis and Martellos 2008), and on stone cultural heritage (Nimis et al. 1992). In particular, 60 thalli per species for each site were selected and treated: (A) on local sandstone masonry blocks at Industria, (B) on sandstone (Macigno sandstone from Lunigiana) blocks, and the adjacent mortar, at the amphitheatre of Luni, and (C) on the sandstone (Pietra Serena) pavement slabs of the monumental Fontana dell'Isola in the Boboli Gardens, at approx. 50 cm from the fountain water.

2.2 Biocide application

Biocides were applied by a professional restorer (site A) or under his supervision (sites B and C). Each biocide was prepared following the manufacturer's instructions (Table 1, including biocide abbreviations) and applied, (i) using a paint-brush and (ii) with a cellulose poultice, using similar quantities of biocide and after having moistened the thalli with sprayed water (Fig. S1). The applied cellulose poultice was kept covered with a cotton fabric for four hours and then gently removed with a small spatula. After the four hours, all brush- and poultice-treated thalli were gently washed with water. The solvents recommended for biocide dilution (water, acetone, White Spirit) were also separately tested. Bottled water with low salt contents was used for all experiments, to avoid any salt-induced reduction in biocide efficiency (Caneva et al. 1996). Three thallus replicates per species per biocide per application method were examined. Daily meteorological data (air temperature, relative humidity, rainfall) for the week preceding and the three weeks following the biocide application at the three sites (A, October 2015; B, April 2016; C, May 2016) were obtained from nearby monitoring stations: A, Verolengo station (ARPA Piemonte 2016), B, Luni station (ARPA Liguria 2016), C, Firenze-Lamma station (ARPA Toscana 2016).

2.3 Lichen vitality measurements

Chlorophyll *a* fluorescence measurements (Chl_aF) were carried out *in situ* one day before (T_0), and one (T_1) and 20 (T_{20}) days after the biocide treatments, using a Handy-PEA fluorimeter (Plant Efficiency Analyser, Hansatech instruments Ltd., Norfolk, England). Analyses were performed early in the morning on dark-adapted moistened thalli, previously sprayed with bottled water and covered overnight with a black cotton fabric. Fifteen minutes before each measurement, thalli were again sprayed and covered. Five measurements were taken on each thallus, positioning the sensor head, equipped with three light emitting diodes (LED), at 90° over its surface and avoiding, in the case of *P. muralis*, areas covered by apothecia. Chl_a fluorescence was induced by a red light (peak at 650 nm) and data recorded after a saturating light pulse of 1 s (Malaspina et al. 2014). Chl_aF increases from F_0 , when all the reaction centres (RCs) of Photosystem II (PSII) are open, to F_M , when all the RCs of PSII are closed. The maximum quantum efficiency of PSII, that is F_v/F_M (where $F_v = F_M - F_0$), a temperature-independent parameter of Chl_aF emission, was used to check the vitality of the thalli (Tretiach et al. 2012).

The analysis of F_V/F_M was combined with a wider evaluation of the OJIP transient, the polyphasic transient exhibited by Chl_aF when plotted on a logarithmic time scale, and of F_0 values. The shape of the OJIP curves is informative on the structure and function of the photosynthetic apparatus (mostly related to PSII) (Malaspina et al. 2014), while F_0 is related to the chlorophyll contents of the light harvesting complex (Baruffo and Tretiach 2007). In site B, additional parameters of the OJIP analysis, including the number of reaction centres (RC), the energy flux trapped by the reaction centres (TR) and the energy flux dissipated as heat (DI), were also considered as indirectly informative on the structure and function of the photosynthetic apparatus upon exposure to stress factors (Malaspina et al. 2014, 2015). All these data were referred to excited cross sections (CS) of the examined lichen, determined by the area of the thallus subjected to the light impulse emitted by PEA (Malaspina et al. 2014).

At site A, Chl_aF measurements were also performed 180 days after the biocide treatments (T180) and combined with epifluorescence observations of hand-made cross-sectioned thalli, carried out under a Nikon Eclipse 300 microscope. Quality and quantity of the fluorescence emitted by photobiont cells, spatially informative on the vitality of the photobiont layer (e.g. Pinna et al. 2012), were evaluated, and the data interpreted using an ordinal scale on the relative abundance of viable (red coloured) and devitalized (appearing white) cells.

At site C, the analysis of Chl_aF in the photobiont of *P. muralis* was combined with analysis of ergosterol content in the mycobiont. Ergosterol is indeed the main sterol of the mycobiont plasma membranes and its content is correlated with basal respiration rates and cell membrane integrity (Sundberg et al. 1999). Analyses were performed as previously described by Vannini et al. (2016). In brief, thallus fragments (100 mg) were homogenized for 10 min in 99% ethanol, and the extracts were shaken in the dark at 25 °C for 30 min, then vortexed and centrifuged at 10,000 g for 20 min. The resulting supernatant was immediately analyzed by HPLC (Hitachi 665A-12 with LC Controller L-5000) using a Phenomenex C18 column (150 x 4.6 mm²; particle size 5 µm) at a flow rate 0.8 mL/min and isocratic elution with methanol as mobile phase. Total analysis time was 15 min. Absorbance at 280 nm was measured with a UV detector (Jasco 875/UV). A standard curve was prepared ranging 1–200 mg ergosterol from Sigma-Aldrich (USA) dissolved in 1 mL of ethanol. Two replicates were measured for each sample.

2.4 Statistics

Generalized Linear Models (GLMs) were applied for each lichen species to describe the effects of the treatments on photobiont vitality (F_V/F_M). For each model we set biocide (water vs. different biocides), time (T0 vs. T1, T20), application method (brush vs. poultice), and site (site C vs. sites A, B) as fixed factors. Second level interactions between biocide, time, application method and site were also considered. GLM analyses were carried out with R (R Development Core Team 2010), using glmer function of lme4 package (Bates et al. 2014). A factorial ANOVA analysis was performed to detect significant differences in F_V/F_M according to the different predictors (biocide, time, application method, site). Calculations were performed using the software package Statistica Version 8.0 (StatSoft, Tulsa, OK).

For each study case, significant differences in F_V/F_M related to time and respect to a threshold (arbitrarily fixed at $F_V/F_M = 0.15$, as discussed in section 4.2) were analyzed by means of ANOVA

with post-hoc Tukey's and t-test, respectively, using SYSTAT 10.2 ($P < 0.05$ as significant). For each study case, decreases of F_0 at T1 and T20, with respect to T0, significantly higher than 80%, were assessed by means of ANOVA with post-hoc t-test ($P < 0.05$ as significant). Data on the other parameters of the OJIP analysis and ergosterol contents were analyzed by means of ANOVA with Tukey's post-hoc test.

230

231 3. Results

GLM tests (Table 2) indicated, for each species, rather uniform F_v/F_M values before the biocide treatments. Slightly higher values characterized *P. muralis* at site A, and lower values occasionally characterized individuals of *V. nigrescens* (in particular, the individuals used to assay BR and WS at site C): however, these values were still within the expected range of variability in viable thalli of this species (Speranza et al. 2012). When treated with water (control), both species did not show variations of F_v/F_M according to time (T0 vs. T1 and T20). Significant differences upon application of all biocides and organic solvents were evident at T1 and T20 (DN, PV, BT, BR, LI, WS, AC \times T1, T20). UA did not affect F_v/F_M , while GL only affected *P. muralis* at T20. Biocide application with cellulose poultice was more effective than brushing in reducing F_v/F_M .

For each species, the range of variability observed in the predictor estimates calculated for the biocide and solvent treatments (at T20: from -0.51 to -0.24 in *P. muralis*, and from -0.38 to -0.15 in *V. nigrescens*) indicated some further differences in their effectiveness. Figures 1 and 2 show F_v/F_M values for the two species at T1 and T20, and percentage decrease in F_0 values with respect to T0, respectively. Only in a few cases did the significant decrease of F_v/F_M with respect to controls correspond to the complete zeroing of the parameter (i.e. 0.00). However, for both species, all biocides (DN, PV, BT, BR, LI) and the two organic solvents (WS, AC) were able to induce, at least in some study cases (application method \times site), a decrease in F_v/F_M below the threshold fixed at 0.15, putatively indicative of the loss of vitality of the photobionts (as discussed in section 4.2).

In *P. muralis*, the decrease of F_v/F_M at T1, including some values below 0.15, was associated with a moderate decrease in F_0 ; while at T20, most F_v/F_M values below 0.15 were associated with a strong decrease in F_0 ($>80\%$ in 82% of cases). In other cases, similarly strong F_0 decreases at T20 were associated with slight signals of F_v/F_M recovery from T1 to T20 (at site C), and, occasionally, with minor lowering of F_v/F_M .

In *V. nigrescens*, the relationship between F_v/F_M values below 0.15 and the strong decrease in F_0 ($>80\%$) was restricted to a more limited set of cases (mostly the poultice treatments at site A). In some others, as described for *P. muralis*, strong decreases in F_0 were related to a moderate lowering of F_v/F_M or, at site C, to slight signals of recovery. Differently, at site B, values of F_v/F_M below 0.15 were associated with relatively poor decreases in F_0 (30-60%).

OJIP transients (Fig. 3) clarified that in all cases the strong decreases of F_v/F_M (<0.15) and/or F_0 ($>80\%$) reflected a substantial loss of vitality of the photobiont. At site C, those slight increases of F_v/F_M from T1 to T20, simulating a partial recovery of both the species (e.g. for BR and LI), depended on ground noise of Chl_aF around zero (Fig. 3C, D). The low percentage decrease of F_0 in *V. nigrescens* at site B was associated with lower initial (T0) values than in other sites (Fig. 3B).

265 In *P. muralis* thalli which displayed strong decreases of F_V/F_M (<0.15) and/or F_0 ($>80\%$)
 266 parameters, the number of reaction centres (RC_0/CS) and the trapped excitons (TR_0/CS) dropped
 267 already at T1 and did not recover at T20 (Table S3). The heat dispersion (DI_0/CS) increased at T1,
 268 and then remarkably dropped at T20. Other thalli displayed a gradual and less pronounced decrease
 269 of RC_0/CS and TR_0/CS ; they also displayed a relative DI_0/CS increase, which, however, was not
 270 followed by dropping at T20.

271 Thalli of *V. nigrescens* strongly affected in F_V/F_M and F_0 also showed a fast drop of RC_0/CS and
 272 TR_0/CS (with the exception of DN samples, for which the parameters dropped at T20) and the
 273 increase of DI_0/CS at T1, which however mostly recovered initial low values at T20 (Table S4). In
 274 thalli displaying no or minor variations in F_V/F_M and F_0 , parameters RC_0/CS , TR_0/CS and DI_0/CS
 275 were not significantly affected.

276 Epifluorescence observations run on thalli from site A at T180 also displayed some differences
 277 between the two species in the photobiont response to biocides (Table S5). Thalli of *P. muralis*
 278 which showed a decrease of F_V/F_M values below 0.15 did not exhibit any living photobiont cell. By
 279 contrast, in *V. nigrescens*, even the thalli with F_V/F_M below 0.15, and a decrease of F_0 by 98-99%,
 280 still showed some residual viable cells in the lower part of the photobiont layer. In parallel, for both
 281 the species, analyses carried out at T180 showed that results evaluated at T20 were mostly reliable
 282 indicators of the long-term response of the thalli to the biocide treatments: only in one out of the 40
 283 treatments (AC applied to *P. muralis* by brush), F_V/F_M values at T20 and T180 displayed a different
 284 position with respect to the threshold fixed at 0.15.

285 To evaluate the consistency of treatment effects across the different sites, the strong decreases of
 286 F_V/F_M (<0.15) and/or F_0 ($>80\%$) were considered indicative of the loss of photobiont vitality (while
 287 residual vitality microscopically observed in *V. nigrescens* was disregarded). At least for some
 288 “biocide \times application method” cases, results obtained at the three sites showed differences (Table
 289 3), possibly due to contrasting environmental conditions. A potential influence of meteorological
 290 conditions preceding, during and following the biocide application (Table S6 in Supplementary
 291 Materials) was considered. Biocide application in site C was performed during a rainy day, and
 292 surfaces were provisionally protected with a plastic canopy and a tarpaulin. Precipitation rates in the
 293 weeks preceding and following the biocide application were rather comparable for the three sites,
 294 while air temperature (T) and humidity (RH) showed relatively slight, but significant (ANOVA,
 295 $P<0.05$) differences, with T in site A $\leq B \leq C$ and RH in A $\geq B \geq C$.

296 Finally, ergosterol content (Fig. 4), although limited to *P. muralis* at site C, reflected the damage
 297 endured by the lichen mycobiont, and suggested further patterns of variability in the potential
 298 effectiveness of the different biocidal approaches (biocide \times application method). Significant
 299 decreases with respect to the water controls were detected for biocides PV, BR and LI and the
 300 organic solvent WS, their effect also depending on the application method. Moreover, a decrease in
 301 ergosterol content was observed for thalli treated with UA when applied with cellulose poultice.

302

303 4. Discussion

Review of the literature suggested that the same active principle may have different levels of biocidal performance in relation to different intrinsic (concentrations, solvents, solution pH, duration of the application) and extrinsic (nature and conservation of the substrate, colonization extent, microclimate) parameters (Caneva et al. 1996, 2008). In the case of lichens, early empirical evaluations (see Caneva et al. 1996) have been poorly supported with quantitative comparative evaluations of intra- and interspecific variability through different case studies.

In this work, we showed the sensitivity of *P. muralis* and *V. nigrescens* to treatments with five widely used biocides, replicated on sandstone surfaces at three heritage sites. Analyses of Chl_aF, quantified as vitality of the photobiont (Tretiach et al. 2010, 2012), demonstrated a significant efficacy of all products, including biocides and their organic solvents, against both species. However, the investigation also displayed for each treatment different levels of efficacy against the two species and/or across the different sites, exposed to slightly different meteorological conditions in days following the biocide applications, and specific patterns of residual vitality.

4.1 Performance and timing of biocidal applications

The tested products are known to exert different biocidal mechanisms: quaternary ammonium compounds, including benzalkonium chloride (DN, PV), interfere with biological membranes by changing their structure and permeability; isothiazolinones (BT, BR, LI) oxidate thiol-containing cytoplasmic and membrane-bound compounds, yielding metabolic inhibition (Denyer and Stewart 1998). Biocides rapidly (T1) reduced the functionality of the photosynthetic process in both tested species, while limited effects (T20) were observed for GL on *P. muralis*, and almost no effect for UA. In laboratory experiments, the same commercial glyphosate solution, at the same concentration (3 times higher than the highest suggested dose), determined a zeroing of F_v/F_m in *Xanthoria parietina* (Vannini et al. 2016), suggesting that different lichen species can differently tolerate this herbicide, which targets a key enzyme (5-enolpyruvyl-shikimate-3-phosphatesynthase, EPSPS) involved in the production of the aromatic aminoacids (phenylalanine, tyrosine and tryptophan), or that, alternatively, laboratory conditions may not properly mimic glyphosate applications on stone materials *in situ*. UA, which was effective to control the growth of other biodeteriogens, such as cyanobacteria and microcolonial fungi (Gazzano et al. 2013), did not affect the *Trebouxia* photobionts of *P. muralis*, a lichen which produces UA (Smith et al. 2009). No effects of UA were also observed on the photobiont partners of *V. nigrescens*, (e.g. *Diploshphaera* sp.; Thus et al. 2011), which does not secrete lichen secondary metabolites. On the other hand, effects of UA on the mycobiont of *P. muralis* suggest a potential role of the secondary metabolite in autoallelopathic processes, poorly explored for lichens and certainly far from being exploitable to control lichens on stone cultural heritage.

Remarkably, we observed a significant effect of pure organic solvents (AC, WS) in the inhibition of photosynthetic processes of both the species. A different AC tolerance of different lichen species was already known (Solhaug and Gauslaa 2001): in laboratory experiments, the time of immersion in AC required to zero F_v/F_m in different lichen species ranged from few hours, compatible to our field treatments, to hundreds of hours (Solhaug and Gauslaa 2001). A higher level of tolerance was attributed to *Trebouxia*-bearing species displaying a high drought resistance, as AC may exert a negative effect by extracting residual water from the dried thalli (Solhaug and Gauslaa 2001). However, AC does not easily pass or destroy the membranes when cells are desiccated (Solhaug

and Gauslaa 2012), while AC was here applied to wet thalli. Accordingly, *P. muralis* and *V. nigrescens* appeared similarly affected by AC, and comparable effects were also observed upon WS treatments.

Biocides were applied after wetting thalli and during humid seasons (Autumn in site A, Spring in sites B and C), when significant rain events, as expected in (sub-)Mediterranean areas, similarly occurred in all the experimental sites, and high values of RH% were generally recorded (see Table S6). Although seasonal variations are known for F_v/F_m in lichens (Baruffo and Tretiach 2007), measurements at T0 generally indicated an overall healthy state of thalli before the biocide application. Effective biocidal effects were already recognizable at T1, in terms of F_v/F_m , RC_0/CS and TR_0/CS decreases, and only for some brush applications of biocides dissolved in water significant decreases were observed later, from T1 to T20. Increased DI_0/CS values at T1 also indicated a fast biocidal activity, as photobionts rapidly attempted to increase controlled de-excitation processes, as thermal energy dissipation, to avoid oxidative damage related to an affected electron flow in the photosynthetic apparatus, as reported under other stress condition (Malaspina et al. 2015).

Since early empirical observations, more noticeable effects of biocides have been recognized when they were applied to wet thalli or when rain events followed their application, while effects have also been observed after some months when biocides were applied in arid seasons (Nimis and Salvadori 1997). When poikilohydric organisms, like lichens, are dehydrated, their structures and macromolecules are protected by glass-like matrices and can tolerate extreme conditions (Tretiach et al. 2012; Fernandez-Marin et al. 2013), including biocide application (Alstrup 1992). Accordingly, the slow-rate effect observed in this study for water-dissolved biocides applied by brush likely depends on the fact that thalli were more rapidly air-dried than those treated with poultices and remained active for a shorter period after the treatment. Moreover, water retention by the porous sandstone lithologies may have contributed to absorb and dilute the brush-applied, water-dissolved biocides (Caneva et al. 1996), limiting a rapid effect.

The application method did not only influence the timing, but, in general, also the biocide performance, with the highest efficacy mostly detected for poultice applications being reasonably explained by an increased contact time between biocide, hydrated thalli, and sandstone substrates (Nugari and Salvadori 2008; Pinna et al. 2012). This may increase rapid effects (observed at T1), but also successive ones, by enhancing biocide retention within substrate, and possibly limiting its washing out under rainy conditions (Young et al. 1995; Caneva et al. 1996; Cameron et al. 1997). In our work, this was evident for the applications of biocides dissolved in water, while minor divergence between the effects of brush and poultice applications was detected for those prepared with organic solvents. Reduced efficacy and recovery were observed when solvents alone were applied by brushing, likely because of the rapid evaporation of the products.

382

383 4.2 *Chl_aF* measures and specific residual vitality

384 The effect of different biocides was associated with different persistence of thallus remnants on the
385 stone surfaces or penetrating structures within the substrate after the mechanical cleaning (de los
386 Ríos et al. 2012). However, scarce information exists to evaluate whether lichen recolonization

387 depends on the arrival of new propagules dispersed by external populations (Favero-Longo et al.
388 2014; Morando et al. in press), or whether mechanical cleaning enhances the spreading of living
389 remnants of partially killed thalli, as documented during attempts to remove thalli without previous
390 devitalization (Seaward 2004, 2015). Cases of recolonization of restored surfaces by lichen
391 communities different from those occurring before the treatments suggest external inputs
392 (Nascimbene et al. 2009). Nevertheless, in other cases, the persistence of lichen remnants after
393 cleaning was correlated with short-term re-increases of lichen cover (e.g. 16 months after the
394 application of isothiazolones combined with benzalkonium chloride or other active principles, in
395 Cámara et al. 2011).

396 In our investigation, the analysis of F_V/F_M was combined with OJIP transients and F_0 variations
397 with the aim of offering a straightforward information on the death of lichen thalli. A threshold of
398 F_V/F_M values reflecting dead photobionts has not been explicitly stated in literature, although dips
399 below 0.100-0.200 often reflect dead material (e.g. Solhaug and Gauslaa 2001; Speranza et al.
400 2012; Tretiach et al. 2012). Accordingly, we accounted for a threshold at $F_V/F_M=0.150$, strictly
401 associated with a dropping of the number of reaction centres (RC_0/CS) and trapped energy fluxes
402 (TR_0/CS).

403 An overview on the OJIP shapes at T20, with a focus on the starting base fluorescence of the curve
404 (F_0), combined with the quantification of the F_V/F_M parameter, helped to check for the residual
405 vitality of the biocide treated thalli. In particular, a strong % decrease of F_0 values (threshold
406 tentatively fixed at -80%), associated with the flattening of curve and the dropping of RC_0/CS and
407 TR_0/CS parameters, appears an additional marker of death. Slight F_V/F_M recoveries, as those
408 observed for both the species in site C, appear irrelevant (as related to measuring noises) when
409 calculated on flat transients with zeroed F_0 values. F_0 variations are primarily related to chlorophyll
410 contents of the light harvesting complex (Baruffo and Tretiach 2007), which are fatally or, at least,
411 severely affected when flat and zeroed curves are calculated.

412 In the light of these discussed Chl_aF parameters, we can finally consider (at least for the poultice
413 applications) that all biocides, and the organic solvents, were effective in killing both the species at
414 least in one of the sites (see Table 4). However, *P. muralis* and *V. nigrescens* were not
415 strongly/fatally affected in 14% and 24%, respectively, of poultice applications of biocides and
416 organic solvents, and displayed different sensitivity to the same treatment (i.e. significant decrease
417 of F_V/F_M and/or F_0 below the thresholds, or not) in 29% of examined cases (biocide \times site; see
418 Table 3). Moreover, epifluorescence observations carried out at T180 suggested some higher
419 resistance for *V. nigrescens*, as some residual healthy photobiont cells were observed in the lower
420 part of the photobiont layer, even in thalli for which both F_V/F_M and F_0 decreased below the fixed
421 thresholds. Accordingly, *V. nigrescens* already showed more resistance to cleaning treatments than
422 another compared species [*Lecidella stigmata* (Ach.) Hertel & Leuckert] after the application of
423 Biotin R (de los Ríos et al. 2012). Thalli of *P. muralis* are thicker than those of *V. nigrescens*, and
424 also display a thicker cortex. However, the very tightly packed, short-celled hyphae of the
425 paraplectenchymatous cortex of *V. nigrescens*, with melanin in the uppermost layers, may
426 determine a lower permeability with respect to the different cortex type of *P. muralis* (cone-cortex;
427 see Büdel and Scheidegger 2008), covered by the hydrophobic, but organic solvent-soluble usnic
428 acid (Smith 2009).

429 Differences across the three sites in the sandstone substrates, possibly related to different physical
430 and chemical properties as suggested by the extreme variability between and within each sandstone
431 type (e.g. Franzini et al. 2007; Fratini et al. 2015), may primary account for the efficacy variability
432 observed for certain treatments. Different porosity, capillary water absorption capacity and mineral
433 composition of different sandstones can indeed affect the quantity of biocide absorbed by the
434 substrate **and its effective life span in a bioactive form** (Young et al. 1995; Cameron et al. 1997).
435 The substrate chemical composition also influences the performance of some biocides, including
436 the quaternary ammonium salts (Caneva et al. 1996). In parallel, different meteorological conditions
437 of each site in the days following the biocide applications could also account for some variability
438 (Nimis and Salvadori 1997). However, cases of poor efficacy were randomly distributed rather than
439 clearly related to any of the heritage sites. Relevance of the variability in T, RH, and sandstone
440 properties between sites on the biocidal mechanism of each product may be hypothesized (Caneva
441 et al. 1996), but should be tested on a wider set of heritage sites, and is beyond the aim of this work.
442 Influences of other environmental conditions, including microclimate differences (Caneva et al.
443 2008), but also biocide-specific population resistances, should be also worth of investigation.

444 Accordingly, waiting for further research to address reliable models to predict the suitability of a
445 biocide against certain species in certain environmental conditions, species- and site-specific
446 calibration of biocidal strategies is necessary. Such calibration would likely exclude the usage of
447 products, concentrations or application methods which may not show the desirable killing efficacy,
448 and would strongly reduce the dispersal of fragments with residual vitality during the mechanical
449 procedures which follow the chemical treatment. If biocides are applied to wet thalli, their efficacy
450 can be confirmed within few weeks (T20) by Chl_aF measurements. Pilot biocide assays appear thus
451 compatible with the time pressure which often characterizes restoration interventions. On the other
452 hand, our results highlighted how the different biocides may also differently affect the mycobiont
453 (as shown by ergosterol content), suggesting the opportunity of conducting controlled experiments
454 on the potential recovery trends on the medium and long term of both the symbiotic partners and
455 their joined influence on the recolonization potential of the different lichen species.

456

457 **5. Conclusions**

458 On the basis of quantitative Chl_aF measurements, our work showed that (a) different biocidal
459 approaches (product × application method) may affect the vitality of lichens at a heritage site,
460 however with different efficacy against each species. Moreover, our findings indicate that (b) the
461 efficacy of a biocidal treatment against a lichen species cannot be assumed to be consistent across
462 different heritage sites. Such complexity suggests that if a biocide approach is planned to manage
463 lichens on a stone surface, *in situ* pilot assays to calibrate biocidal treatments on the particular study
464 case (species × site) should be run.

465

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476

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625

626 **Tables**

627 Table 1 - Biocides and solvents applied in the experiments

Commercial product	Producer		Active principle	Solvent	Recommended concentration	Used concentration
Water	W	-	-	-	-	-
Glifene SL	GL	Chmiberg-Diachem (Caravaggio, Italy)	Glyphosate [i.e. N-(phosphonomethyl)glycine; 30-40%] in water	Water	0.003 - 0.011%	0.04%
DesNovo	DN	Bresciani S.r.l. (Milano, Italy)	Benzalkonium chloride (i.e. alkyl dimethyl benzyl ammonium chloride; 10%) in water	Water	0.5 - 10%	2.00%
Preventol® R180	PV	Lanxess (Köln, Germany)	Benzalkonium chloride (i.e. alkyl dimethyl benzyl ammonium chloride; approx. 80%) + isopropyl alcohol (2%) in water	Water	1 - 2%	2.00%
BiotinT	BT	C.T.S. S.r.l. (Altavilla Vicentina, Italy)	N-octyl-isothiazolinone (7.0-10.0%) + didecyl-dimethyl ammonium chloride (40.0-60.0%) + formic acid (2.0-2.5%) + isopropyl alcohol (15.0-20.0%)	Water	1 - 3 %	2.00%
White Spirit	WS	Sinopia S.A.S. (Torino, Italy)	-	-	-	-
BiotinR	BR	C.T.S. S.r.l.	N-octyl-isothiazolinone (3-5%) + 3-iodoprop-2-ynyl N-butylcarbamate (10-25%) in diethylene glycol butyl ether	White Spirit (100%)	3 - 5%	3.00%
Acetone (≥99.9% for HPLC)	AC	Sigma-Aldrich (St. Luis, MO, USA)	-	-	-	-
Lichenicida 464	LI	Bresciani S.r.l.	4,5-Dichloro-2-octyl-4-isothiazolin-3-one (25.0-<40.0%) + 3-Iodo-2-propynyl N-butylcarbamate (12.5-<15.0%) + 2-Octyl-4-isothiazolin-3-one (0.06-<0.10%) + benzyl alcohol (40.0-<60.0%)	Acetone (100%)	1 - 2%	2.00%
Usnic Acid	UA	Sigma-Aldrich	Usnic acid [i.e. 2,6-Diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3,(2H,9bH)-dione; powder]	Acetone (1%)	-	0.0005%

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631 Table 2 - Summary of the Generalized Linear Models examining the effects of predictors on F_V/F_M,
632 written as F_V/F_M~Biocide × Application method × Site × Time. *, P<0.05; **, P<0.01; ***,
633 P<0.001.

Predictor	<i>P. muralis</i>				<i>V. nigriscens</i>			
	Estimate	Standard error	t value	P value	Estimate	Standard error	t value	P value
(Intercept)	5.02E-01	2.58E-02	19.443	<2E-16 ***	5.91E-01	2.57E-02	22.987	<2E-16 ***
Biocide-AC	5.11E-02	3.53E-02	1.449	0.1481	-4.15E-02	3.54E-02	-1.171	0.24228
Biocide-BR	5.69E-02	3.53E-02	1.612	0.1076	-6.97E-02	3.54E-02	-1.968	0.04958 *
Biocide-BT	2.87E-02	3.53E-02	0.814	0.4159	-3.59E-02	3.54E-02	-1.012	0.31187
Biocide-DN	3.68E-02	3.48E-02	1.056	0.2914	-3.61E-02	3.54E-02	-1.018	0.30907
Biocide-GL	4.87E-02	3.53E-02	1.381	0.168	-4.53E-02	3.54E-02	-1.28	0.20108
Biocide-LI	5.01E-02	3.53E-02	1.419	0.1566	-5.82E-02	3.54E-02	-1.644	0.1007
Biocide-PV	-5.92E-05	3.53E-02	-0.002	0.9987	-4.72E-02	3.54E-02	-1.332	0.18346
Biocide-UA	3.57E-02	3.53E-02	1.011	0.3125	-3.65E-02	3.54E-02	-1.031	0.30322
Biocide-WS	3.65E-02	3.53E-02	1.033	0.3019	-9.94E-02	3.54E-02	-2.806	0.00521 **
Time T1	6.66E-02	3.48E-02	1.915	0.056	9.99E-03	3.45E-02	0.29	0.77214
Time T20	1.81E-02	3.48E-02	0.52	0.6035	-4.36E-02	3.54E-02	-1.23	0.21914
Application-Poultice	-5.95E-02	9.18E-03	-6.487	2.07E-10 ***	-7.32E-02	9.35E-03	-7.834	2.76E-14 ***
Site A (Industria)	8.65E-02	1.12E-02	7.724	5.92E-14 ***	7.57E-03	1.14E-02	0.662	0.50804
Site B (Luni)	-7.84E-03	1.13E-02	-0.694	0.4883	-1.77E-02	1.15E-02	-1.543	0.12355
Biocide AC : Time T1	-4.61E-01	4.99E-02	-9.238	<2E-16 ***	-4.41E-01	5.01E-02	-8.815	<2E-16 ***
Biocide BR : Time T1	-5.99E-01	4.99E-02	-12.001	<2E-16 ***	-4.19E-01	5.01E-02	-8.366	5.70E-16 ***
Biocide BT : Time T1	-2.76E-01	4.99E-02	-5.533	5.02E-08 ***	-2.76E-01	5.01E-02	-5.516	5.51E-08 ***
Biocide DN : Time T1	-1.97E-01	4.92E-02	-4.004	7.16E-05 ***	-1.36E-01	5.01E-02	-2.724	0.00666 **
Biocide GL : Time T1	-9.75E-03	4.99E-02	-0.195	0.8451	-2.34E-02	5.01E-02	-0.467	0.64043
Biocide LI : Time T1	-5.92E-01	4.99E-02	-11.863	<2E-16 ***	-4.29E-01	5.01E-02	-8.557	<2E-16 ***
Biocide PV : Time T1	-3.35E-01	4.99E-02	-6.715	4.98E-11 ***	-3.05E-01	5.01E-02	-6.091	2.21E-09 ***
Biocide UA : Time T1	2.04E-02	4.99E-02	0.408	0.6831	1.28E-02	5.01E-02	0.256	0.79834
Biocide WS : Time T1	-5.08E-01	4.99E-02	-10.186	<2E-16 ***	-2.70E-01	5.01E-02	-5.4	1.03E-07 ***
Biocide AC : Time T20	-3.32E-01	4.99E-02	-6.66	7.07E-11 ***	-3.60E-01	5.07E-02	-7.093	4.39E-12 ***
Biocide BR : Time T20	-5.08E-01	4.99E-02	-10.195	<2E-16 ***	-2.79E-01	5.07E-02	-5.496	6.14E-08 ***
Biocide BT : Time T20	-2.36E-01	4.99E-02	-4.741	2.76E-06 ***	-2.61E-01	5.07E-02	-5.141	3.89E-07 ***
Biocide DN : Time T20	-2.56E-01	4.89E-02	-5.238	2.38E-07 ***	-2.61E-01	5.07E-02	-5.137	3.98E-07 ***
Biocide GL : Time T20	-1.12E-01	4.99E-02	-2.239	0.0256 *	2.28E-02	5.07E-02	0.449	0.65339
Biocide LI : Time T20	-4.93E-01	4.99E-02	-9.893	<2E-16 ***	-3.79E-01	5.07E-02	-7.466	3.59E-13 ***
Biocide PV : Time T20	-3.37E-01	4.99E-02	-6.748	4.06E-11 ***	-3.14E-01	5.07E-02	-6.181	1.30E-09 ***
Biocide UA : Time T20	-3.59E-02	4.99E-02	-0.719	0.4724	4.52E-03	5.07E-02	0.089	0.929
Biocide WS : Time T20	-2.77E-01	4.99E-02	-5.557	4.41E-08 ***	-1.54E-01	5.07E-02	-3.043	0.00246 **

Table 3 - Synoptic comparison of biocide efficacy at T20 against the photobionts of *P. muralis* and *V.nigrescens* at the three sites. For each species, biocide treatments which determined devitalization (†, *, or †*) in all the heritage sites are marked in bold. Biocide abbreviations are reported in Table 1.

	Brush			Poultice		
	Site A (Industria)	Site B (Luni)	Site C (Boboli)	Site A (Industria)	Site B (Luni)	Site C (Boboli)
<i>P. muralis</i>						
W	=	=	=	=	=	=
GL	=	-	-	=	=	-
DN	=	-	-	*	†	-
PV	*	-	-	*	†	†*
BT	=	-	=	*	†*	*
WS	-	-	=	-	†*	*
BR	†*	†*	*	†*	†*	†*
AC	*	=	-	=	†*	*
LI	†*	†*	*	†*	†*	*
UA	=	-	=	-	-	-
<i>V. nigrescens</i>						
W	=	=	=	=	-	=
GL	=	=	-	=	=	-
DN	-	†	-	†*	-	-
PV	†	=	=	†*	†	*
BT	*	=	=	†*	†	-
WS	=	=	=	†*	†	*
BR	-	=	*	=	†	†*
AC	†*	†	=	†*	†	-
LI	†*	†	*	†	†	*
UA	=	=	-	=	=	=

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641 =, F_V/F_M did not significantly decrease with respect to T0, and F_0 decreased with respect to T0 < 80%

642 -, F_V/F_M significantly decreased with respect to T0, but it was not significantly lower than the threshold fixed at 0.15,
643 and F_0 decreased < 80%

644 †, F_V/F_M significantly decreased with respect to T0 and it was significantly lower than 0.15, but F_0 decrease < 80%

645 *, F_0 decreased > 80%, but F_V/F_M was not significantly lower than 0.15

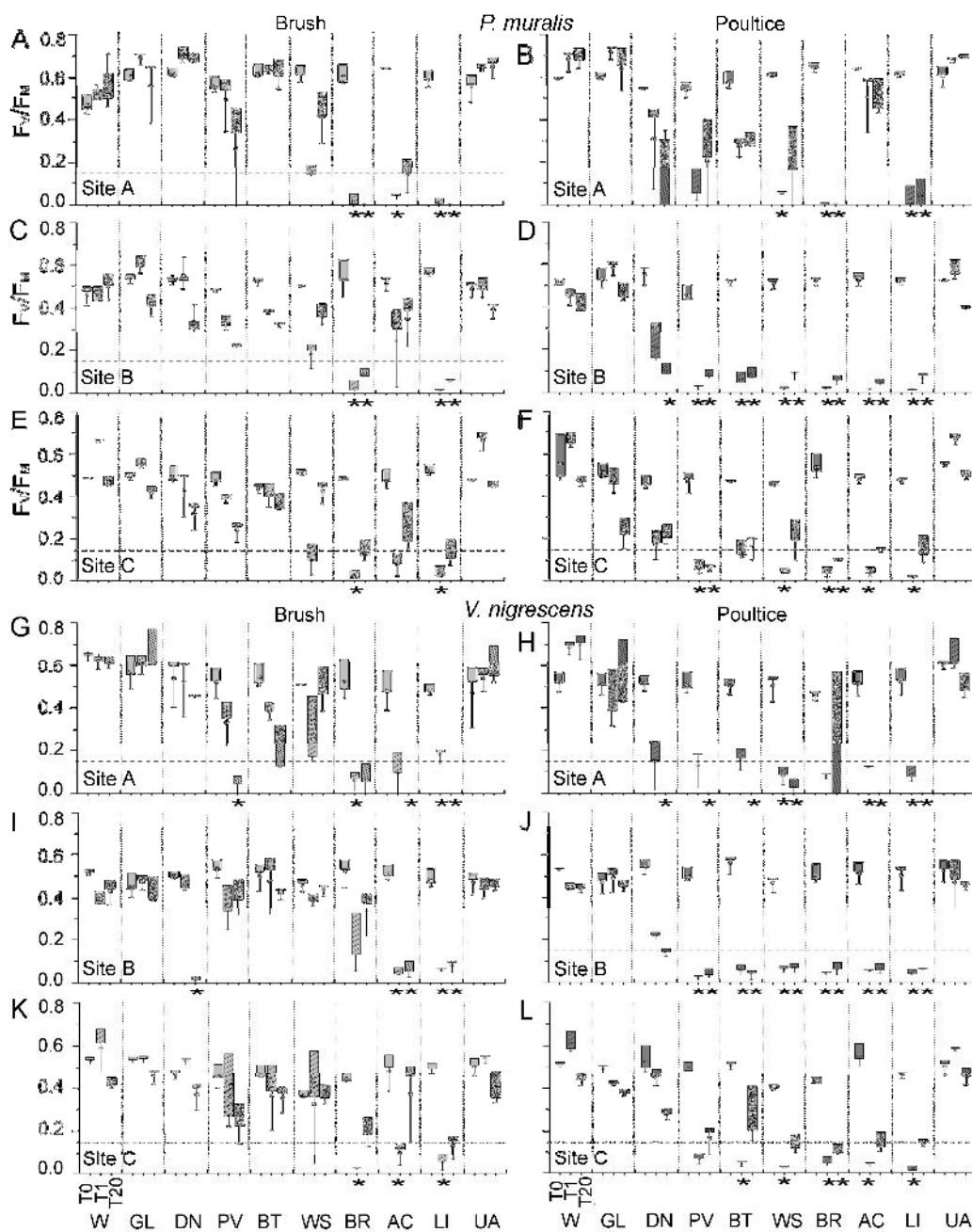
646 †*, F_V/F_M was significantly lower than 0.15, and F_0 decreased > 80%

647

648 **Figure captions**

649 Fig. 1 - Maximum quantum efficiency of Photosystem II photochemistry (F_v/F_M) in thalli of
650 *Protoparmeliopsis muralis* (A-F) and *Verrucaria nigrescens* (G-L) measured *in situ* (Site A,
651 Industria; site B, Luni; site C, Boboli) before (T0), and 1 (T1) and 20 (T20) days after the biocide
652 application with brush or using a cellulose poultice. Biocide abbreviations are reported in Table 1.
653 For each case study (biocide \times application method \times site), F_v/F_M values (mean \pm SD) which are
654 significantly lower than a threshold fixed at 0.15 (horizontal dashed line) are marked (*; ANOVA,
655 t-test; $P < 0.05$). Overview tables of measures on *P. muralis* and *V. nigrescens*, including a statistical
656 comparison for each study case of F_v/F_M values at T0, T1 and T20, are reported in Supplementary
657 Materials S1 and S2, respectively.

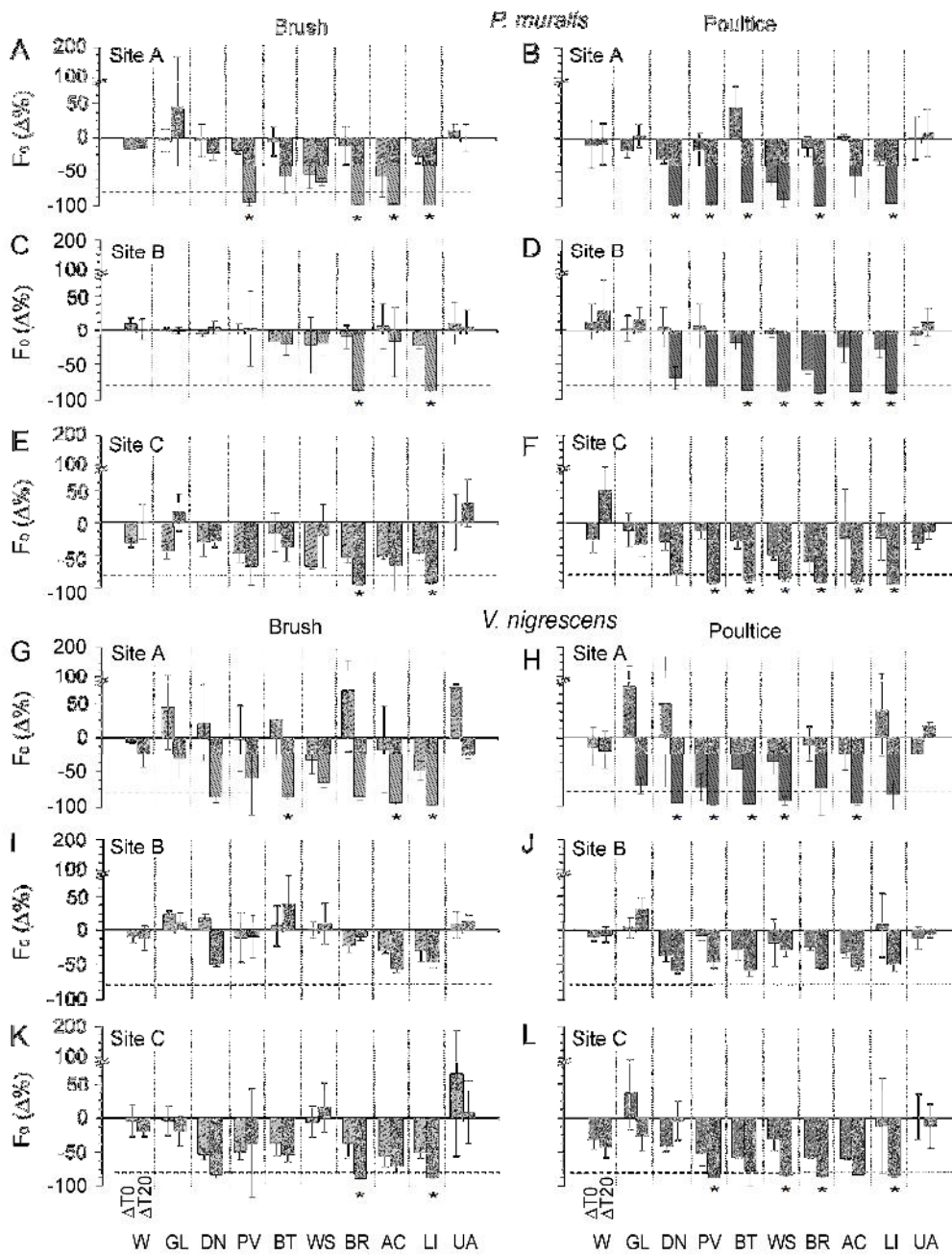
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661 Fig. 2 - Variation of F_0 values ($\Delta\%$ at T1 and T20 with respect to T0, i.e. 1 and 20 days after the
662 biocide application with brush or using a cellulose poultice) in thalli of *P. muralis* (A-F) and *V.*
663 *nigrescens* (G-L) examined in site A, Industria, site B, Luni, and site C, Boboli. Biocide
664 abbreviations are reported in Table 1. For each case study (biocide \times application method \times site),
665 variations of F_0 (mean \pm SD) which are significantly lower than -80% (horizontal dashed line) are
666 marked (*; ANOVA, t-test; $P < 0.05$).

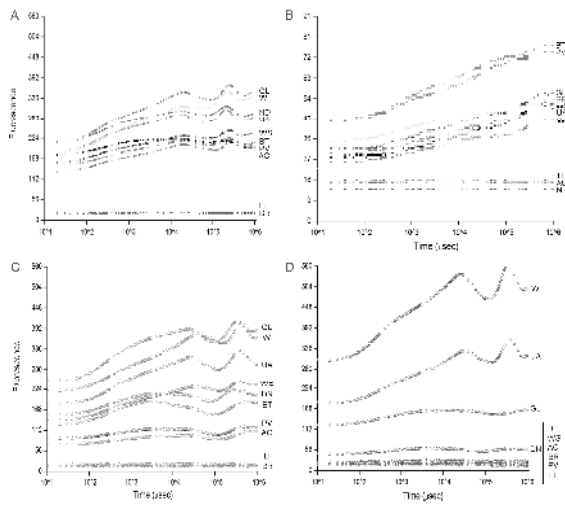


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Fig. 3 - OJIP fluorescence transients at T20 (exemplifying set) of *P. muralis* (A) and *V. nigrescens* (B) in site B (Luni) after the biocide application with brush, and of *P. muralis* in site C (Boboli) after the biocide application with brush (C) or using a cellulose poultice (D). Each transient is the average of the data obtained for the different replicates (5 measures for 3 thalli) of each case study. Biocide abbreviations are reported in Table 1.

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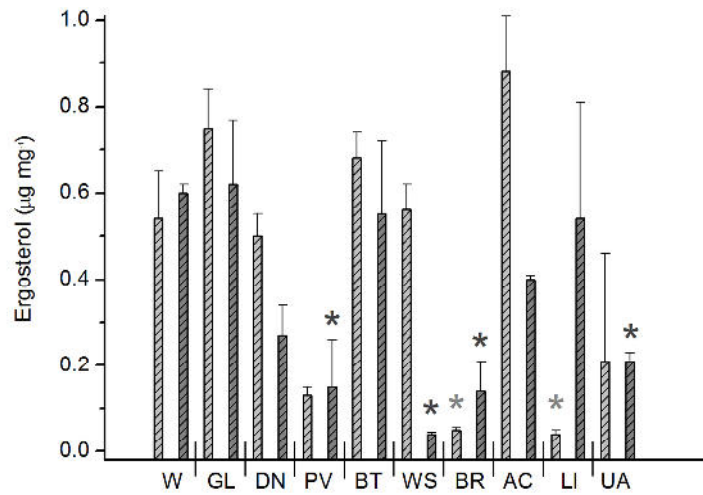


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678 Fig. 4 – Ergosterol content in thalli of *P. muralis* in site C (Boboli) 20 days after the biocide
 679 application (T20) by brush (light grey columns) or with cellulose poultice (dark grey columns).
 680 Data are expressed as mean value \pm SD. For each application method, significant decreases in
 681 ergosterol following the biocide treatments, with respect to contents in thalli sprayed with water
 682 only, are indicated (*, ANOVA, Tukey's test; $P < 0.05$). Biocide abbreviations are reported in Table
 683 1.



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